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CIN85 regulates dopamine receptor endocytosis and governs behavior in mice

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 29 September 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. Please let me first apologise once again for the long delay in getting back to you with a decision - I do realise that you have been waiting for quite some time, and I really appreciate your patience. As I told you, we experience some difficulty in finding three appropriate and available referees, and also a delay in receiving their reports. In fact, we are still awaiting the third report, but at this stage it is not clear if/when we will receive this. I am therefore taking a decision - to invite a revision of your manuscript - based on the two reports we have to hand. Obviously, if the third report comes in, we will forward it on to you, and may also ask you to respond to this reviewer's comments.

As you will see from the reports appended below, both referees express interest in the phenotype of the CIN85 knockout mouse, and in its apparent role in regulating dopamine receptor trafficking and function. However, while referee 2 raises only relatively minor concerns, referee 1 finds that further in vivo work on the regulation of dopamine receptor trafficking would be required before the manuscript would be suitable for publication. The suggested experiments should not involve additional genetics, and therefore I hope it will be possible to address these concerns during the time frame of the revision (normally three months). However, should you need an extension - up to a maximum of 6 months - please just let me know and we should be able to accommodate this. I would also draw your attention to the comments of referee 2 concerning the link between the

regulation of dopaminergic signalling and the behavioural hyperactivity. It would be important to make it very explicit in the text that the link is currently correlative not causal.

In the light of the referees' positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of both reviewers. As I said above, I will be in touch again if and when the third report arrives. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This article reports on a novel function of α-CIN85 in regulating dopamine D2 receptors uptake in striatal neurons. In particular, α-CIN85 knockout animals display a mild locomotor hyperactivity and do not respond to very low doses of haloperidol, a D2 specific antagonist. It is proposed that α-CIN85 might be required for the internalization of D2 receptors. Authors also report on increased dopamine and its metabolites levels in striatal extracts, which is at odds with a putative increase of D2 receptors at the membrane.

The data contained in the article although sounds require more in depth in vivo analyses.

For example, the number of 3H-Spiperone binding sites should be analyzed on striatal extracts and not only on primary cultures, before and after quinpirole treatment of mice.

Similarly, a pharmacological characterization of α-CIN85 mutants should be performed, using agonists and antagonists of D1 and D2 receptors.

Authors should propose a mechanism to explain why dopamine levels are increased, are D2 receptor sites increased also in the substantia nigra and VTA? If so, they should find a decrease of dopamine in the striatum not an increase. Are other molecules, inside the dopaminergic system, affected by loss of α-CIN85?

All biochemical experiments are based on the use of a commercial anti-D2 antibody, which detects a sharp D2 receptor's specific band at ~50 KDa. Most laboratories working in the field find a smear of D2 receptor specific bands of approximate size around 70KDa. The use of extracts from D2 receptor knockout mice would validate the findings shown in this article.

In conclusion, this article is potentially interesting but needs more work in support of a functional interaction of α-CIN85 and D2 in vivo.

Referee #2 (Remarks to the Author):

Simokawa and colleagues characterize a CIN85 knockout mouse. They identify locomotor hyperactivity in the intact animals and describe reduced endocytosis of D2 receptors in cortical

neurons cultured from knockouts. Based on increased dopamine levels in CIN85 mutants the authors propose that the hyperactivity phenotype is a consequence of inhibited D2 endocytosis.

CIN85 is a multifunctional protein implicated in endocytosis of various proteins such as EGF receptors, and in binding to numerous cellular proteins including Cbl and ubiquitin, based on extensive in vitro and structural study. The authors state that the present study is the first to study the role of this protein in the intact mouse. This makes the study intrinsically interesting, and the proposed function of CIN85 in D2 receptor endocytosis is new.

An intrinsic weakness of such a study, given the large number of potential binding partners and functions of CIN85, is that it is difficult to be sure that the behavioral hyperactivity observed is solely a consequence of increased surface D2 receptors. Nevertheless this is consistent with the data so is not an unreasonable proposal.

My only specific critical concern has to do with the demonstration that CIN85 functions in D2 receptor endocytosis. 1. Can the authors test the statistical significance of the difference observed between wild type and mutant animals. From the error bars it looks probably significant, but this could be tested more formally (such as by non-paired Student's t-test).

2. Can they show that D2 endocytosis is rescued in cultures by CIN85 transfection?

Addtional Editorial Correspondence 08 October 2009

I have finally received the last referee's report (copied below) on your manuscript - apologies again for the long delay with this. As you will see, referee 3 expresses significant interest in your study, but does also raise a number of serious concerns that would need to be addressed in your revision. In particular, I would draw your attention to the concerns regarding the definitiveness of the data to pertaining to the proposed dopamine receptor internalisation defect (point 6 and associated comments), and also to point 10, concerning the data presented in Figure S7, which I agree is pivotal to the conclusions of the manuscript. In addition, the referee highlights a number of statements in the text that need to be toned down (or backed up by experimental data). I would therefore ask that you respond to all the points raised by this referee when preparing your revised manuscript - a number of the major concerns can be addressed by modifications to the text, but further experimental analysis will also be necessary. As I mentioned before, we can offer you an extension on the revision deadline should you need it - please just let me know if you think this would be helpful.

Once again, I am sorry for the delay in getting this report, but I hope you agree that it provides constructive comments for your revision.

I look forward to receiving your revised manuscript.

Yours sincerely,

Editor
EMBO Journal

REFEREE REPORT:

Referee 3 comments:

The manuscript by Shimokawa et al describes the production and characterization of a gene targeted

mouse model that lacks two brain-specific isoforms of the cbl interacting protein of 85kDa (CIN85-l and CIN85-xl). CIN85 also known as SH3KBP1 is an adaptor protein, which has previously been shown to have an important role in endocytic cargo sorting. Previous in vitro studies by the authors and others have demonstrated a strong role for CIN85 in the endosomal-lysosomal ligand dependent sorting of tyrosine kinase receptors, such as the epidermal growth factor receptor (EGFR). The aim of this study was to identify a function for CIN85 in vivo, specifically in the central nervous system. CIN85 is expressed as 8 different isoforms with different expression patters from multiple promoters and via alternative splicing. The knockout CIN85Δex2 described in this manuscript was generated by targeting exon 2 for deletion, which resulted in loss of expression of CIN85 isoforms expressed from promoter 1, mainly CIN85-xl and CIN85-l. These two isoforms the authors demonstrate are also the predominant brain-specific isoforms of CIN85. The main body of the manuscript describes the molecular, electrophysiological and behavioral characterization of the CIN85Δex2 mouse.

Cellular evidence is presented that CIN85-xl and CIN85-l are expressed in many brain regions and are present in the dendritic spines of rat hippocampal cultured neurons and postsynaptic fractions from mouse striatum and that these isoforms are lost in whole brain extracts of the CIN85Δex2 mouse. From an extensive series of physiological and behavioral studies the authors summarize that the CIN85Δex2 mice display hyperactive behavior as evidenced by increased locomoter and exploratory activities. Furthermore they conclude this phenotype explains the increased energy uptake, higher lean mass and lower fat content that also characterizes the CIN85Δex2 mouse. The authors then present evidence to show there are no defects in hippocampal synaptic plasticity that could explain the observed behavioral phenotype. In contrast, the authors demonstrate defects in the striatal dopaminergic system, specifically increased levels of dopamine and its metabolites in striatal tissue, which they try to attribute to a defect in dopamine- induced D2 type dopamine receptor endocytosis. Finally the authors suggest this is due to a defect in recruitment of the endocytic protein endophilin to D2 dopamine receptors in the absence of CIN85-xl and CIN85-l in the striatum of these mice, that ultimately reduces D2DR signaling in the striatum and loss of the normal striatal dopaminergic regulation of locomotion.

The major claims of this manuscript are of general interest to the readers of EMBO and several novel findings are reported. However this reviewer is not convinced the data fully substantiates the molecular mechanisms suggested by the authors to explain the phenotype of the CIN85Δex2 mouse. Several major problems with the manuscript were identified plus numerous specific issues that I have outlined below.

SPECIFIC COMMENTS

Major comments:

1. There are several unsubstantiated comments in the abstract.

i) "CIN85Δex2 animals display abnormally high levels of dopamine and dopamine receptors in the straitum" - The authors do not present data showing comparative measurements of dopamine receptor levels in wild type versus CIN85Δex2 striatal tissue.

ii) "CIN85 localizes to the postsynaptic compartment of striatal dendritic spines" - The authors do not present data showing CIN85 is in striatal dendritic spines -see comment 3.

iii) " In response to dopamine stimulation, absence of striatal CIN85 causes insufficient recruitment of the endocytic regulator endophilin to D2 dopamine receptors (D2DR) and ultimately decreased D2DR endocytosis." - The authors do not present data showing endophilin recruitment in response to dopamine, therefore the authors cannot make this statement of a suggestive mechanism. In addition the data does not show that there is a defect in D2DR endocytosis per se; see comment 6.

2. There are several unsubstantiated comments in the discussion. i) There is no data to support the statement " CIN85 is enriched in the postsynaptic compartment of dendritic spines" ii) There is no data presented here to support the statement " we have identified a crucial role of CIN85 in the organization of endocytic complexes". Data showing a reduction in the binding of endophilin to

D2DR does not consistute "organization of a complex". The data as is, one could argue suggests CIN85 stabilizes endophilin binding. Most damagingly the data does not support the molecular mechanism proposed in the discussion to explain the phenotype.

3. The data in Figure 1B clearly shows CIN85 localization in dendrites and dendritic spines of primary cultured hippocampal neurons. There is several references in this manuscript to data showing CIN85 localization in dendritic spines of striatal neurons, but there is no data to demonstrate this. To make this claim, the authors need to provide data to prove this. Synapses are located on spines and on shafts of dendrites, therefore the data presented in Fig1D cannot be used as a substitute for this missing data. In addition data demonstrating co-localization of CIN85 with D2DR in striatal neurons is missing and would easily address the discussion point raised in p17, line 7.

4. The authors provide no data to substantiate their claim (page 8, line 7) that there is a clear knockout-specific gene expression profile phenotype?

5. The statement on page 8, paragraph 2 relating to explorative behavior in a novel environment states that "both female and male CIN85Δex2 mice showed significantly increased activities, as compared to wild-type littermates (Figure 3B)". The data as presented in Figure 3B is not separated into male versus female comparisons of each genotype as in Figure 3A. Therefore this statement cannot be made, unless the data was determined but is not presented here. In which case the male versus female statement should be removed and the data as presented reported clearly in the results section and the phrase "data not shown" n added for clarification.

6. The data (Figure 5) the authors present to demonstrate D2DR endocytosis is defective does not substantiate this claim. The assay utilized in Figure 5B and C measures only the amount of receptor at the cell surface in their case at 1 hour after the addition of agonist. This amount will be the sum of the receptors that have not internalization during this time and those that have internalized and recycled back to the cell surface. CIN85 is a protein that has previously been implicated in the endosomal sorting of receptors to the lysosomal pathway. Therefore an equally likely explanation for the observed increase in D2DR number at the cell surface of CIN85Δex2 striatal neurons is that in the absence of CIN85, D2DR receptors are no longer sorted to the lysosome and are recycled back to the cell surface. The authors need to address this possible alternative experimentally, before they can conclude the defect is at the step of membrane internalization.

7. The author's description of the radioligand-binding assay in the text of the results (12, para2) is confusing. Clarification of the assay used would be achieved by replacing the phrase "in which the amount of 3H-Spiperone.....was analyzed in the membrane fraction" to "in which the binding of 3H-Spiperone.....to membrane fractions was analyzed". Also in the Figure legend to 6A the authors should align their conclusion to what the assay is actually testing, i.e not movement, but the sum of movements (see comment 5 above).

8. The weak data discussed in points 5 and 6 would also be strengthened by presentation of data showing relative steady state amounts of total receptor and steady state levels of surface expressed receptor. This type of analysis would help to explain their findings. In addition it would address the comments made in the discussion p16 line 11 and p16, para2, line 2, which at present are unsubstantiated.

9. The data in Figure 6 and S6 does not provide evidence to support the claim of "defective recruitment of Endophilin to D2 receptors in CIN85Δex2 mice". The immunoprecipitations were performed on non-stimulated tissue, therefore the argument cannot be made that the binding of any of these components is a recruitment process. If indeed Endophilin is recruited to D2DR upon ligand binding then the appropriate data needs to be presented to support this statement. That said the quantitative data presented in FigS6 that demonstrates there is a deficit in endophilin binding to D2DR in these mice, is important and should be incorporated into Figure 6 as it is integral to the findings presented. Again as mentioned above statistical analysis of the reduction in binding needs

to be done and the actual numbers +/- etc need to presented in the main body of the manuscript, not an

approximate number. Also be consistent with the nomenclature used for the material that was tested between the figure legend and the text.

10. The data in Fig S7 is pivotal to connecting the defects observed in D2DR membrane trafficking with an in vivo deficit in D2DR signaling in these mice. This data should be integral to the manuscript and not within the supplementary material. In addition the data needs to be more rigorously quantified than as it is presented at the moment. Specifically what assay was performed, how it was quantified and the appropriate statistical tests need to be performed on the data.

11. The discussion is verbose and too long; please shorten. The discussion of the differential binding of the two dynamin isoforms to either D1 or D2 dopamine receptors is irrelevant, given that no alteration in dynamin binding was found. Also the last paragraph is interesting but irrelevant to this study, given that no learning defects were observed with these mice. Suggest removing at minimum these two parts of the discussion.

12. How CIN85 is recruited to D2DR is an important question given what is known from the literature of the mechanisms of action of CIN85 with respect to tyrosine kinase receptors. CIN85 is a cbl binding protein, therefore it is surprising that the possible association of Cbl to D2 dopamine receptors was not addressed in this manuscript. Have the authors addressed this experimentally? In addition data exploring whether cbl recruits CIN85 to D2DR one would also expect to be addressed here. These are obvious analyses that this reviewer feels are missing from the manuscript and even if the results are negative some reference should be made to the findings in the text of this manuscript. Furthermore inclusion of the preliminary data presented in figure S8 in the discussion section is inappropriate, particularly as no conclusion can be derived form this figure that helps to explain the findings of this manuscript; suggest removing.

Minor Comments:

1. Many grammatical errors noted, check carefully before re-submission.

i) Odd phrasing p6, para 2 "We could accordingly confirm", suggest re-phrasing.

ii) P7, para 2,change "'neither" of the transcripts' to "none".

iii) P8, line 2 "phenotypes not necessarily are apparent" change.

iv) P8, line 3 "to an extensive" drop "an".

v) P11, 6th line form bottom "Even though also" drop also.

...etc...

2. Fig 1a, There is a faint band in all lanes at around 75kD, is this a CIN85 isoform or an artifact, please explain.

3. There are quality issues in Figure 1, specifically with Fig 1C&D. In the text the data is described as presented in 1D, therefore please present the data for Fig1C to be consistent with Fig1D fractions. Also to note in Fig1C the figure quality is poor with the hand written labels shown and the lane alignment is off between the top and bottom panels. Also in Fig1D, what are the other bands?

4. Figure 2D, the second CIN85 isoform, which I assume is CIN85-l is mislabeled.

5. In Figure 3 the stars noting significance probabilities are misplaced, please correct the figure.

6. Figure 5B, in the legend please state the name of the D1DR dopamine agonist used. Approximate numbers are inappropriate add specifics +/- and statistics applied. Add stats also to the figure. Also in the text add a statement to denote that you used AMPAR-GluR2 as a control.

7. As above for figure 5C, in the legend please state the name of the D2DR dopamine agonist used. Approximate numbers are inappropriate add specifics +/- and statistics applied. Add stats also to the figure. In the text add a statement to denote that you used AMPAR-GluR2 as a control. In addition the numbers stated in the figure legend are inconsistent with those stated in the text.

8. With figures 6B and C there are quality issues. In 6B the migration of CIN85 seems quite different between blots, especially on the IP of endophilin. Also in the IP of Dynamin there is an additional upper band, can the authors make a mention of what this is, isoform of CIN85 or artifact please. In 6C the western blot of PSD95 is of very poor quality, please replace. In addition there is an inconsistency with the material used for the IP (6C) in the figure legend with the text, please correct.

9. p13, last sentence of para 1, odd phrasing for the description of the IP from the knock-out tissue, please re-phrase.

1st Revision - Authors' Response 26 April 2010

Referee #1 (Remarks to the Author):

This article reports on a novel function of α-CIN85 in regulating dopamine D2 receptors uptake in striatal neurons. In particular, α-CIN85 knockout animals display a mild locomotor hyperactivity and do not respond to very low doses of haloperidol, a D2 specific antagonist. It is proposed that α-CIN85 might be required for the internalization of D2 receptors. Authors also report on increased dopamine and its metabolites levels in striatal extracts, which is at odds with a putative increase of D2 receptors at the membrane.
The data contained in the article although sounds require more in depth in vivo analyses.

1. For example, the number of 3H-Spiperone binding sites should be analyzed on striatal extracts and not only on primary cultures, before and after quinpirole treatment of mice.

We agree that it is important to analyze the number of [^{3}H]spiperone binding sites in the striatal extracts in mice, not only in primary neuronal cultures. According to the referee's advice, we added an experiment analyzing whether the number of [^{3}H]spiperone binding sites are changed by treatment with the D2 dopamine receptor agonist quinpirole, based on previous papers (Culver and Szechtman, 2003; Culver et al, 2008). The revised Figure 5A shows that [^{3}H]spiperone binding is 45% and 73% higher in saline-treated CIN85$^{\Delta ex2}$ knockout mice and quinpirole-sensitized CIN85$^{\Delta ex2}$ knockout mice, respectively, than in wild-type mice treated with saline. Detailed description and comments concerning the results in revised Figure 5A were added in the Results section of our revised manuscript.

2. Similarly, a pharmacological characterization of α-CIN85 mutants should be performed, using agonists and antagonists of D1 and D2 receptors.

We have performed additional pharmacological characterization of CIN85$^{\Delta ex2}$ mutant mice, analyzing the effect of quinpirole, a D2-selective dopamine receptor agonist, and of haloperidol, a pharmacological inhibitor of dopamine receptors, on the behavior of CIN85$^{\Delta ex2}$ knockout mice. When exposing mice to low doses of quinpirole or haloperidol, we found CIN85$^{\Delta ex2}$ knockouts to be clearly less sensitive to the locomotor modulating effects of these drugs, as compared to wild-type littermates (Revised Figures 5B and 5C), suggesting alterations in D2 dopamine receptor levels and/or D2 mediated signaling in the CNS of CIN85$^{\Delta ex2}$ mice. Details are explained in the Results section of the paper. The results from the [^{3}H]spiperone binding in Point 1 above also show that D2

dopamine receptor levels in the striatum are increased in CIN85$^{\Delta ex2}$ knockouts compared to wild-type animals. We also performed an additional pharmacological experiment with a low dose of the D1-selective dopamine receptor antagonist SCH23390, which exerted comparable effects on both CIN85$^{\Delta ex2}$ knockout mice and wild-type littermates (Figure 5D). This latter experiment confirmed our conclusion from the *in vitro* experiments, that CIN85 plays a role in D2-mediated, but not in D1-mediated processes. Detailed description and comments concerning the results in revised Figure 5 were added in the Results section of our revised manuscript.

3. Authors should propose a mechanism to explain why dopamine levels are increased, are D2 receptor sites increased also in the substantia nigra and VTA? If so, they should find a decrease of dopamine in the striatum not an increase. Are other molecules, inside the dopaminergic system, affected by loss of α-CIN85?

We don't have certain experimental data to explain why the dopamine content has increased in the striatum of CIN85$^{\Delta ex2}$ knockout mice, but we think as outlined below. The dopamine molecules released from the presynaptic terminals bind to dopamine receptors on the postsynaptic membrane through the synaptic cleft in the striatum. Dopamine-receptor complexes are internalized normally (wild-type mice) by endocytic proteins and then dopamine molecules are degraded by monoamine oxidase B (MAO-B). However, in CIN85$^{\Delta ex2}$ knockout mice the enzymatic degradation of dopamine molecules in postsynaptic neurons is inhibited by the defect of internalization/endocytosis. Therefore, the dopamine content has increased in the striatum of CIN85$^{\Delta ex2}$ knockout mice. If distinction between kinetic analysis of the presynaptic cells and that of the postsynaptic cells will become possible, we can solve this important issue.

4. All biochemical experiments are based on the use of a commercial anti-D2 antibody, which detects a sharp D2 receptor's specific band at ~50 KDa. Most laboratories working in the field find a smear of D2 receptor specific bands of approximate size around 70KDa. The use of extracts from D2 receptor knockout mice would validate the findings shown in this article.

In the original experiment we performed Western blotting using an anti-D2DR antibody obtained from Santa Cruz Biotech. The antibody is a mouse monoclonal antibody raised against amino acids 1-50 of D2DR of human origin. According to the referee's comment, to confirm of D2DR specific band, we performed Western blotting using an anti-D2DR antibody raised against another epitope. The antibody (AB5084P, Millipore) that we used in the revised experiment is a rabbit polyclonal antibody raised against a 28 amino acid peptide within the 3rd cytoplasmic loop of the human D2DR. We thus have confirmed the specificity of D2DR bands. These new results are shown in revised Figure 4B.

In conclusion, this article is potentially interesting but needs more work in support of a functional interaction of α-CIN85 and D2 in vivo.

We feel that our new data provide extensive further evidence for a functional interaction between CIN85 and D2 dopamine receptors *in vivo*.

Referee #2 (Remarks to the Author):

Simokawa and colleagues characterize a CIN85 knockout mouse. They identify locomotor hyperactivity in the intact animals and describe reduced endocytosis of D2 receptors in cortical neurons cultured from knockouts. Based on increased dopamine levels in CIN85 mutants the authors propose that the hyperactivity phenotype is a consequence of inhibited D2 endocytosis.

CIN85 is a multifunctional protein implicated in endocytosis of various proteins such as EGF receptors, and in binding to numerous cellular proteins including Cbl and ubiquitin, based on extensive in vitro and structural study. The authors state that the present study is the first to study the role of this protein in the intact mouse. This makes the study intrinsically interesting, and the proposed function of CIN85 in D2 receptor endocytosis is new.

An intrinsic weakness of such a study, given the large number of potential binding partners and functions of CIN85, is that it is difficult to be sure that the behavioral hyperactivity observed is solely a consequence of increased surface D2 receptors.

Nevertheless this is consistent with the data so is not an unreasonable proposal.

1. My only specific critical concern has to do with the demonstration that CIN85 functions in D2 receptor endocytosis. Can the authors test the statistical significance of the difference observed between wild type and mutant animals. From the error bars it looks probably significant, but this could be tested more formally (such as by non-paired Student's t-test).

According to the referee's comment, we performed statistical analysis (by ANOVA and Duncan multiple range test for posthoc between group comparisons) between the data of the endocytosis/internalization of D2DR in wild-type and that of CIN85$^{\Delta ex2}$ knockout mice. We have shown these results in revised Figures 4B and 5A.

2. Can they show that D2 endocytosis is rescued in cultures by CIN85 transfection?

We have given careful consideration to the comment from the Referee. Since the transfection efficiency of cDNA into primary neurons is very low, this experiment seems to be difficult. We have therefore unfortunately not performed the rescue experiment.

Referee 3 comments:

The manuscript by Shimokawa et al describes the production and characterization of a gene targeted mouse model that lacks two brain-specific isoforms of the cbl interacting protein of 85kDa (CIN85-l and CIN85-xl). CIN85 also known as SH3KBP1 is an adaptor protein, which has previously been shown to have an important role in endocytic cargo sorting. Previous in vitro studies by the authors and others have demonstrated a strong role for CIN85 in the endosomal-lysosomal ligand dependent sorting of tyrosine kinase receptors, such as the epidermal growth factor receptor (EGFR). The aim of this study was to identify a function for CIN85 in vivo, specifically in the central nervous system. CIN85 is expressed as 8 different isoforms with different expression patters from multiple promoters and via alternative splicing. The knockout CIN85Δex2 described in this manuscript was generated by targeting exon 2 for deletion, which resulted in loss of expression of CIN85 isoforms expressed from promoter 1, mainly CIN85-xl and CIN85-l. These two isoforms the authors demonstrate are also the predominant brain-specific isoforms of CIN85. The main body of the manuscript describes the molecular, electrophysiological and behavioral characterization of the CIN85Δex2 mouse.

Cellular evidence is presented that CIN85-xl and CIN85-l are expressed in many brain regions and are present in the dendritic spines of rat hippocampal cultured neurons and postsynaptic fractions from mouse striatum and that these isoforms are lost in whole brain extracts of the CIN85Δex2 mouse. From an extensive series of physiological and behavioral studies the authors summarize that the CIN85Δex2 mice display hyperactive behavior as evidenced by increased locomoter and exploratory activities. Furthermore they conclude this phenotype explains the increased energy uptake, higher lean mass and lower fat content that also characterizes the CIN85Δex2 mouse. The authors then present evidence to show there are no defects in hippocampal synaptic plasticity that could explain the observed behavioral phenotype. In contrast, the authors demonstrate defects in the striatal dopaminergic system, specifically increased levels of dopamine and its metabolites in striatal tissue, which they try to attribute to a defect in dopamine-induced D2 type dopamine receptor endocytosis. Finally the authors suggest this is due to a defect in recruitment of the endocytic protein endophilin to D2 dopamine receptors in the absence of CIN85-xl and CIN85-l in the striatum of these mice, that ultimately reduces D2DR signaling in the striatum and loss of the normal striatal dopaminergic regulation of locomotion.

The major claims of this manuscript are of general interest to the readers of EMBO and several novel findings are reported. However this reviewer is not convinced the data fully substantiates the molecular mechanisms suggested by the authors to explain the phenotype of the CIN85Δex2 mouse.

Several major problems with the manuscript were identified plus numerous specific issues that I have outlined below.

SPECIFIC COMMENTS

Major comments:

1. There are several unsubstantiated comments in the abstract.

i) "CIN85Δex2 animals display abnormally high levels of dopamine and dopamine receptors in the straitum" - The authors do not present data showing comparative measurements of dopamine receptor levels in wild type versus CIN85Δex2 striatal tissue.

We agree that it is necessary to compare the dopamine receptor levels in striatal tissue from wild-type versus CIN85$^{\Delta ex2}$ knockout mice. According to this advice from the referee and a similar comment from referee #1, we measured the dopamine receptor levels in the striatum as shown in revised Figure 5A by analyzing the number of [^{3}H]spiperone binding sites before and after treatment with the D2DR agonist quinpirole, based on previous papers, in whole striata prepared from both wild-type and CIN85$^{\Delta ex2}$ mice (Culver and Szechtman, 2003; Culver et al, 2008). Revised Figure 5A shows that [^{3}H]spiperone levels are 45% and 73% higher in saline treated CIN85$^{\Delta ex2}$ knockout mice and quinpirole-sensitized CIN85$^{\Delta ex2}$ knockout mice, respectively, compared to wild-type mice treated with saline, suggesting that the D2 dopamine receptor levels are higher in the striatal tissue of CIN85$^{\Delta ex2}$ knockout mice compared to wild-type controls both under normal conditions and following quinpirole sensitizaton. Detailed comments on revised Figure 5A and conclusions were added in the Results and Discussion sections of our revised manuscript.

ii) "CIN85 localizes to the postsynaptic compartment of striatal dendritic spines" - The authors do not present data showing CIN85 is in striatal dendritic spines -see comment 3.

We agree with the reviewer and have performed immunofluorescence studies of primary rat striatal neurons in culture, showing that CIN85 localizes to spine-like structures of dendrites that contain F-actin (Figure 1C). We also find CIN85 co-clustered with D2DRs in punctuate synapse-like structures in primary striatal neurons (Figure 4D). Together, these data suggest a localization of CIN85 to synapses of primary striatal neurons.

iii) "In response to dopamine stimulation, absence of striatal CIN85 causes insufficient recruitment of the endocytic regulator endophilin to D2 dopamine receptors (D2DR) and ultimately decreased D2DR endocytosis." - The authors do not present data showing endophilin recruitment in response to dopamine, therefore the authors cannot make this statement of a suggestive mechanism. In addition the data does not show that there is a defect in D2DR endocytosis per se; see comment 6.

The statement has now been modified as follows throughout the text: "Absence of striatal CIN85 causes insufficient complex formation of endophilins with D2 dopamine receptors (D2DRs) and ultimately decreased D2DR endocytosis". Concerning the defect in D2DR endocytosis, please see the response to comment 6 below.

2. There are several unsubstantiated comments in the discussion.

i) There is no data to support the statement " CIN85 is enriched in the postsynaptic compartment of dendritic spines"

We agree with the reviewer and have modified all statements to “CIN85 localizes post-synaptically in neurons” (or similar) (Figure 1B and Figure 1C).

ii) There is no data presented here to support the statement " we have identified a crucial role of CIN85 in the organization of endocytic complexes". Data showing a reduction in the binding of endophilin to D2DR does not consistute "organization of a complex". The data as is, one could argue suggests CIN85 stabilizes endophilin binding.

We agree with the point and have changed the statement to “we have identified a crucial role of CIN85 in stablilizing the endophilin binding to D2DRs”.

iii) Most damagingly the data does not support the molecular mechanism proposed in the discussion to explain the phenotype.

New data presented in Revised Figures 4 and 5 combined with our previous findings strongly suggest a role of CIN85 in regulating D2 dopamine receptor levels and endocytosis in the striatum and striatal neurons, respectively, which provide, at least in part, an explanation to the apparent hyperactivity phenotype of the CIN85$^{\Delta ex2}$ mice. Molecularly, the reduced complex formation between endophilins and D2DRs in the striatum in the absence of CIN85 provides a mechanism by which the reduction of D2DR endocytosis may occur. The new data, conclusions and discussions in support of this molecular mechanism are presented in the Results and Discussion sections of the revised manuscript.

3. The data in Figure 1B clearly shows CIN85 localization in dendrites and dendritic spines of primary cultured hippocampal neurons. There is several references in this manuscript to data showing CIN85 localization in dendritic spines of striatal neurons, but there is no data to demonstrate this. To make this claim, the authors need to provide data to prove this. Synapses are located on spines and on shafts of dendrites, therefore the data presented in Fig1D cannot be used as a substitute for this missing data. In addition data demonstrating co-localization of CIN85 with D2DR in striatal neurons is missing and would easily address the discussion point raised in p17, line 7.

We agree with the referee and have performed stainings of primary rat striatal neurons in culture, showing that CIN85 localizes to spine-like structures of dendrites in striatal neurons, by co-localization studies with F-actin (Figure 1C). We also show co-localization between CIN85 and D2DR at punctuate synapse-like structures in striatal neurons (Figure 4D). We have carefully modified all statements accordingly.

4. The authors provide no data to substantiate their claim (page 8, line 7) that there is a clear knockout-specific gene expression profile phenotype?

These data have not been included due to space limitations and we have now referred to them as “data not shown”.

5. The statement on page 8, paragraph 2 relating to explorative behavior in a novel environment states that "both female and male CIN85Δex2 mice showed significantly increased activities, as compared to wild-type littermates (Figure 3B)". The data as presented in Figure 3B is not separated into male versus female comparisons of each genotype as in Figure 3A. Therefore this statement cannot be made, unless the data was determined but is not presented here. In which case the male versus female statement should be removed and the data as presented reported clearly in the results section and the phrase "data not shown" n added for clarification.

We have changed the statements to "CIN85$^{\Delta ex2}$ mice showed significantly increased activities, as compared to wild-type littermates (Figure 3B)".

6. The data (Figure 5) the authors present to demonstrate D2DR endocytosis is defective does not substantiate this claim. The assay utilized in Figure 5B and C measures only the amount of receptor at the cell surface in their case at 1 hour after the addition of agonist. This amount will be the sum of the receptors that have not internalization during this time and those that have internalized and recycled back to the cell surface. CIN85 is a protein that has previously been implicated in the endosomal sorting of receptors to the lysosomal pathway. Therefore an equally likely explanation for the observed increase in D2DR number at the cell surface of CIN85Δex2 striatal neurons is that in the absence of CIN85, D2DR receptors are no longer sorted to the lysosome and are recycled back to the cell surface. The authors need to address this possible alternative experimentally, before they can conclude the defect is at the step of membrane internalization.

We have interest in the referee's comments. Previous reports have demonstrated that D1 dopamine receptor (D1DR) rapidly internalizes in response to dopamine and recycles *in vivo* (Ariano MA, et al., *Synapse* 27:313–21, 1997). In contrast, D2DR is generally trafficked to the lysosomal pathway and degraded (Bartlett SE, et al., *Proc Natl Acad Sci USA* 102:11521–6, 2005). From these observations and the referee's comments, we analyzed the amount of D2DR at the cell surface for short time stimulation (5, 15 and 30 min) in addition to the 60 min stimulation by agonist. Revised Figure 4B shows that the levels of cell surface D2DR in striatal neurons from CIN85$^{\Delta ex2}$ knockout mice were 96.5% and 87.5% at 15 and 30 min after dopamine stimulation, respectively, whereas surface levels of D2DR in striatal neurons of wild-type mice at the same time points were 72.3% and 59.7%, respectively. In addition, even at the time (5-15 min after dopamine stimulation) as the D2DR internalization accelerated in wild-type neurons, striatal neurons from CIN85$^{\Delta ex2}$ knockout mice maintained high levels of cell surface D2DR that remained high throughout the time course. Therefore, we conclude that the increased D2DR levels at the cell surface are not due to defective receptor degradation and increased recycling back of D2DR to the cell surface, but rather due to a defect in receptor internalization. Comments and conclusions concerning the results in revised Figure 4B are presented in the Results and Discussion sections of our revised manuscript.

7. The author's description of the radioligand-binding assay in the text of the results (12, para2) is confusing. Clarification of the assay used would be achieved by replacing the phrase "in which the amount of 3H-Spiperone.....was analyzed in the membrane fraction" to "in which the binding of 3H-Spiperone.....to membrane fractions was analyzed". Also in the Figure legend to 6A the authors should align their conclusion to what the assay is actually testing, i.e not movement, but the sum of movements (see comment 5 above).

These statements have been modified according to the referees's comments.

8. The weak data discussed in points 5 and 6 would also be strengthened by presentation of data showing relative steady state amounts of total receptor and steady state levels of surface expressed receptor. This type of analysis would help to explain their findings. In addition it would address the comments made in the discussion p16 line 11 and p16, para2, line 2, which at present are unsubstantiated.

The data showing decreased D2 dopamine receptor endocytosis in striatal neurons in revised Figure 4B and the increased levels of D2 dopamine receptors in the striatum as a whole, presented in revised Figure 5A, together strongly implicate a role for CIN85 in regulation of dopamine receptor endocytosis and thereby dopamine receptor levels in the striatum. Indeed, the increased D2 dopamine receptor levels and stimulation with a D2 agonist promote elevated locomotor activity (e.g. revised Figure 5A).

9. The data in Figure 6 and S6 does not provide evidence to support the claim of "defective recruitment of Endophilin to D2 receptors in CIN85Δex2 mice". The immunoprecipitations were performed on non-stimulated tissue, therefore the argument cannot be made that the binding of any of these components is a recruitment process. If indeed Endophilin is recruited to D2DR upon ligand binding then the appropriate data needs to be presented to support this statement. That said the quantitative data presented in FigS6 that demonstrates there is a deficit in endophilin binding to D2DR in these mice, is important and should be incorporated into Figure 6 as it is integral to the findings presented. Again as mentioned above statistical analysis of the reduction in binding needs to be done and the actual numbers +/- etc need to presented in the main body of the manuscript, not an approximate number. Also be consistent with the nomenclature used for the material that was tested between the figure legend and the text.

We agree and have changed all statements to "decreased complex formation between endophilins and D2DRs in CIN85$^{\Delta ex2}$ mice" (or alike). The data on endophilin binding from former Figure S6 have been incorporated as an integral part of the manuscript in revised Figure 5F with specifics and statistical analyses. The nomenclature in the figure legend and text has been revised.

10. The data in Fig S7 is pivotal to connecting the defects observed in D2DR membrane trafficking with an in vivo deficit in D2DR signaling in these mice. This data should be integral to the manuscript and not within the supplementary material. In addition the data needs to be more rigorously quantified than as it is presented at the moment. Specifically what assay was performed, how it was quantified and the appropriate statistical tests need to be performed on the data.

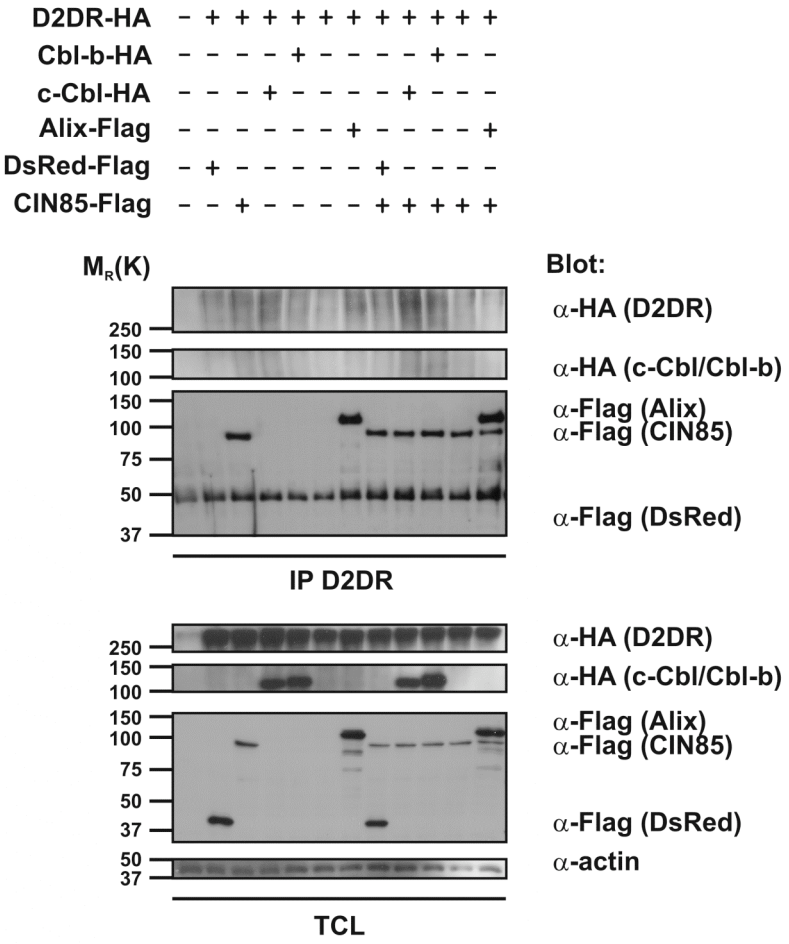
We agree that these data are important and should be an integral part of the manuscript. The data are presented in revised Figure 5C and have been subjected to statistical analyses. Details about quantifications, the assay itself and statistical analyses are described in the Materials and Methods and the Figure 5C legend of our revised manuscript. Our data suggest a reduced sensitivity of CIN85$^{\Delta ex2}$ knockouts to the locomotor suppressant effects of a low dose of haloperidol compared to wild-type mice, in line with our proposed mechanism that D2 dopamine receptor levels are increased in neurons of CIN85$^{\Delta ex2}$ mice. This conclusion has been further substantiated by additional pharmacological experiments - see also the response to Referee #1, Point 2, and revised Figure 5 in the manuscript.

11. The discussion is verbose and too long; please shorten. The discussion of the differential binding of the two dynamin isoforms to either D1 or D2 dopamine receptors is irrelevant, given that no alteration in dynamin binding was found. Also the last paragraph is interesting but irrelevant to this study, given that no learning defects were observed with these mice. Suggest removing at minimum these two parts of the discussion.

These two parts of the discussion have been removed. The discussion has also been shortened and improved as a whole.

12. How CIN85 is recruited to D2DR is an important question given what is known from the literature of the mechanisms of action of CIN85 with respect to tyrosine kinase receptors. CIN85 is a cbl binding protein, therefore it is surprising that the possible association of Cbl to D2 dopamine receptors was not addressed in this manuscript. Have the authors addressed this experimentally? In addition data exploring whether cbl recruits CIN85 to D2DR one would also expect to be addressed here. These are obvious analyses that this feels are missing from the manuscript and even if the results are negative some reference should be made to the findings in the text of this manuscript. Furthermore inclusion of the preliminary data presented in figure S8 in the discussion section is inappropriate, particularly as no conclusion can be derived form this figure that helps to explain the findings of this manuscript; suggest removing.

We have tested the interaction between overexpressed D2DRs and c-Cbl and Cbl-b in HEK293T cells, but could not detect an interaction (see the Western blot below). In the same assay, CIN85 and ALIX interacted with the D2DR. The data concerning p62 have now been described in the main text (Figure S8). We are continuing our search for proteins linking D2DRs and CIN85.



Minor Comments:

1. Many grammatical errors noted, check carefully before re-submission.
i) Odd phrasing p6, para 2 "We could accordingly confirm", suggest re-phrasing.
ii) P7, para 2,change '"neither" of the transcripts' to "none".
iii) P8, line 2 "phenotypes not necessarily are apparent" change.
iv) P8, line 3 "to an extensive" drop "an".
v) P11, 6th line form bottom "Even though also" drop also.
...etc...

These points have been corrected and we have carefully gone through the entire text to correct any further mistakes.

2. Fig 1a, There is a faint band in all lanes at around 75kD, is this a CIN85 isoform or an artifact, please explain.

This is most likely an artifact, given that we generally do not detect such a band in CIN85

Western blots.

3. There are quality issues in Figure 1, specifically with Fig 1C&D. In the text the data is described as presented in 1D, therefore please present the data for Fig1C to be consistent with Fig1D fractions. Also to note in Fig1C the figure quality is poor with the hand written labels shown and the lane alignment is off between the top and bottom panels. Also in Fig1D, what are the other bands?

The description of these experiments has been corrected in the text and the hand written labels have been removed and the panels aligned in revised Figure 1D. The other bands in revised Figure 1E are unspecific bands that appeared in this particular Western blot.

4. Figure 2D, the second CIN85 isoform, which I assume is CIN85-l is mislabeled.

This is right and the labeling of this isoform has now been corrected to CIN85-l.

5. In Figure 3 the stars noting significance probabilities are misplaced, please correct the figure.

The stars noting significance have been adjusted in Figures 3A and 3B.

6. Figure 5B, in the legend please state the name of the D1DR dopamine agonist used. Approximate numbers are inappropriate add specifics +/- and statistics applied. Add stats also to the figure. Also in the text add a statement to denote that you used AMPAR-GluR2 as a control.

According to the referee's advice, the name of D1DR dopamine agonist has been stated in the revised Figure 4C legend (Old Figure 5B). Specifics and statistics have been included in legend and figure and in addition, the control has been mentioned in the text.

7. As above for figure 5C, in the legend please state the name of the D2DR dopamine agonist used. Approximate numbers are inappropriate add specifics +/- and statistics applied. Add stats also to the figure. In the text add a statement to denote that you used AMPAR-GluR2 as a control. In addition the numbers stated in the figure legend are inconsistent with those stated in the text.

The name of D2DR dopamine agonist has been stated in the revised Figure 4B legend (Old Figure 5C) in which the numbers have also been added and corrected according to the referee's comment. Statistics have also been added in the legend and the figure and the control has been mentioned in the text.

8. With figures 6B and C there are quality issues. In 6B the migration of CIN85 seems quite different between blots, especially on the IP of endophilin. Also in the IP of Dynamin there is an additional upper band, can the authors make a mention of what this is, isoform of CIN85 or artifact please. In 6C the western blot of PSD95 is of very poor quality, please replace. In addition there is an inconsistency with the material used for the IP (6C) in the figure legend with the text, please correct.

The molecular weight standards were unfortunately shifted in the blots mentioned and this has now been corrected. The additional band in the dynamin IP may be CIN85-xl, but also an unspecific band. The PSD95 Western blot has been changed and the material used for the assay has been revised.

9. p13, last sentence of para 1, odd phrasing for the description of the IP from the knock-out tissue, please re-phrase.

The statement has been rephrased.

2nd Editorial Decision 11 May 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2009- 72253R.

It has now been seen again by the original referee 3, whose comments are enclosed below. As you will see, he/she is largely satisfied with the revision and now finds the manuscript suitable for publication. However, he/she does raise a couple of minor remaining concerns that it would be good if you could address in a final revision of the text. For point 2, I recognise that you do show the quantitation of the data and error bars, but it would be valuable to state the statistical significance (p value) in the text.

I suggest that the easiest way forward would be for you to send us a revised manuscript text file by email, incorporating these minor changes. We can then upload the new version into the system, and should then be able to accept the manuscript without further delay.

Many thanks for all the effort that went in to the extensive revision of the manuscript - I realise that there was a lot of work involved here to address the referees' concerns. I think you now have a really nice story here and am happy to be publishing it in EMBOJ!

Yours sincerely,

Editor
EMBO Journal

Referee 3 comments:

The revised version of this manuscript is much improved in the writing and the additional experiments address most of my major concerns. The new discussion is also much improved. I have a couple of remaining concerns however, but I think these can be mostly addressed in the writing. That is if the authors agree to the suggestions.

Remaining concerns:

1. In response to original comment #1 and specifically to the unsubstantiated statement in the original abstract "In response to dopamine stimulation, absence of striatal CIN85 causes insufficient recruitment of the endocytic regulator endophilin to D2 dopamine receptors (D2DR) and ultimately decreased D2DR endocytosis".... The authors have addressed this by altering the phrase. However the new statement in the abstract is still not an accurate representation of their data. It still suggests they demonstrate absence of CIN85 interferes with the formation of a dopamine stimulated D2DR-endophilin complex and subsequently D2DR endocytosis. The endocytosis data clearly supports this statement as it was generated in response to dopamine stimulation. However the data analyzing the D2DR-endophilin interactions did not address whether agonist (dopamine) binding stimulates the D2DR-endophilin interaction. Hence invalidating this statement in the abstract. I suggest a suitable distinction should be made about the results in the writing of this statement in the abstract.

2. In response to the original comment 9, the authors indicate they have performed statistical analysis and incorporated this into the text. However the statistical data is still missing, please add.

As this experiment is crucial to the authors proposed molecular mechanism, evidence that the finding is statistically significant needs to be added.

Minor comments:

1. Figure legend 5F change "were prepared as in (D) to as in (E).

2nd Revision – Authors' Response 17 May 2010

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We have changed the abstract according to the reviewer´s comment as follows: "Absence of striatal CIN85 causes insufficient complex formation of endophilins with D2DRs in the striatum and ultimately decreased D2DR endocytosis in striatal neurons in response to dopamine stimulation."

2. In response to the original comment 9, the authors indicate they have performed statistical analysis and incorporated this into the text. However the statistical data is still missing, please add. As this experiment is crucial to the authors proposed molecular mechanism, evidence that the finding is statistically significant needs to be added.

We have added the statistical analysis and p value for Figure 5F in the text and figure legend and exchanged the right panel in Figure 5F according to the referee's comment. The decrease in endophilin binding to D2DRs in CIN85$^{\Delta ex2}$ mutant striata compared to wild-type is indeed significant. The new parts of the text and figure legend are now as follows:

Page 16: Quantification of the binding of endophilin to D2DRs in wild-type and CIN85$^{\Delta ex2}$ striatal synaptosome fractions from four independent experiments showed that the interaction between D2DR and endophilin in CIN85$^{\Delta ex2}$ knockout striata is significantly decreased to about 65% of the interaction observed in wild-type samples (Student's *t*-test, $p < 0.05$) (Figure 5F, middle and right panels).

Page 25: A graph depicting the mean value of the relative endophilin intensities in CIN85$^{\Delta ex2}$ knockout (-/-) compared to wild-type (+/+) mice from the four experiments is shown in the right panel. The value is presented as mean +/- SE. Differences between wild-type (+/+) and CIN85$^{\Delta ex2}$ knockout mice (-/-) were analyzed using a Student's *t*-test, with the level of significance set at $p < 0.05$. +/+: $n = 4$; -/-: $n = 4$.

Minor comments:
1. Figure legend 5F change "were prepared as in (D) to as in (E).

We have changed this in the figure legend.

